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Purification and properties of a new clathrin assembly protein

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A clathrin assembly protein (AP_{180}) has been purified and characterized from coated vesicles of bovine brain. This protein has hitherto escaped detection because in SDS–gel electrophoresis it is obscured by the 180 kd heavy chain of clathrin. Despite the similarity in electrophoretic mobility, AP_{180} differs from clathrin in both its subunit and native mol. wt, as well as hydrodynamic properties, surface charge and tryptic peptide composition. It also appears immunologically distinct from clathrin, since neither a polyclonal antiserum nor a monoclonal antibody, that have been shown to be specific for AP_{180}, cross-react with the heavy chain of clathrin. AP_{180} binds to clathrin triskelia and thereby promotes clathrin assembly into regular polyhedral structures of narrow size-distribution (60–90 nm), reminiscent of the surface coat of coated vesicles. In this respect AP_{180} bears a functional resemblance to the 100–110 kd clathrin assembly polypeptides that have been previously described.

Key words: coated vesicles/clathrin triskelia/assembly proteins/monoclonal antibody

Introduction

In eukaryotic cells clathrin-coated membranes are involved in certain routes of specific membrane traffic such as receptor-mediated endocytosis and the transfer of newly synthesized proteins from the *trans*-face of the Golgi-stacks to terminal destinations, such as lysosomes and secretory granules (Goldstein *et al.*, 1979; Pearse and Bretscher, 1981). Biochemical dissection and reconstitution studies on purified coated vesicle preparations from bovine brain or human placental tissue provided a rudimentary insight into the structural organisation of this organelle. The polyhedral coat of the vesicles is constructed mainly from the 180 kd polypeptide clathrin which in its trimeric form (the clathrin triskelion) constitutes the basic structural unit of the polyhedral network (Ungewickell and Branton, 1981). Tightly bound to clathrin-triskelia are three polypeptides with relative mol. wts of 33 and 36 kd, usually referred to as the light chains of clathrin (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981; Ungewickell, 1983). Exposure of coated vesicles to low concentrations of urea (Schook *et al.*, 1977), alkaline pH in the presence of chelators (Woodward and Roth, 1978) or high concentrations of protonated amines (Keen *et al.*, 1979) causes release of clathrin and other major polypeptides of relative mol. wts of 100–110, 50, 40 and 16.5 kd. Clathrin (R_S ~16 nm) is readily separated by gel filtration from the accompanying proteins which elute close together, at a position corresponding to an average Stokes radius of 7.4 nm. Purified clathrin polymerizes spontaneously at millimolar calcium ion concentrations into polymorphous cage-like structures, that closely resemble the coat of coated vesicles (Keen *et al.*, 1979). In the absence of calcium, however, self-assembly of clathrin triskelia into ordered polyhedral structures is strictly dependent on the presence of fractions containing the 16.5–110 kd components, which are therefore referred to as assembly polypeptides (Keen *et al.*, 1979) or 100 kd proteins (Pearse and Robinson, 1984). Cages reassembled in the presence of assembly proteins differ markedly from the generally larger and less homogeneous structures formed by addition of calcium (Zaremba and Keen, 1983). A subset of the assembly proteins comprising at least three polypeptides between 100 and 110 kd and the 50 kd polypeptide, was recently purified by hydroxyapatite adsorption chromatography and is referred to as the HA-II group of the 100 kd polypeptides (Pearse and Robinson, 1984). Chemical cross-linking experiments suggested that some of the 100 kd polypeptides and the 50 kd polypeptide are associated in a complex of some 300 kd. The assembly proteins of the HA-II group are also capable of inducing the polymerization of clathrin into a homogeneous population of small cages. By contrast, another group of the 100 kd polypeptides (HA-I group) which is also composed of several 100–110 kd polypeptides but lacks the 50 kd polypeptide, gives rise to a mixed population of large (>100 nm) and small reconstituted cages (Pearse and Robinson, 1984).

Earlier evidence suggested that the 100–110 kd components of the assembly proteins might also be responsible for attaching clathrin to the membrane of coated vesicles (Unanue *et al.*, 1981). More recently a direct association between polypeptides of the HA-II group and a *trans*-membrane protein (the mannose-6-phosphate receptor) was indeed demonstrated (Pearse, 1985).

Based on these observations the assembly proteins have been surmised to control not only the size of coated vesicles but also, and perhaps more importantly, the selection of membrane proteins for intracellular transport. Progress in further characterization of the assembly proteins has been limited by the difficulty of purifying single species to homogeneity. In the present study the purification and properties of a new clathrin assembly protein from bovine brain, which escaped previous detection because of its failure to resolve from the clathrin heavy chain in SDS–PAGE is described.

Results

Fractionation of assembly proteins

Clathrin and other peripheral membrane proteins were liberated from the membrane of purified coated vesicles with 0.5 M Tris and fractionated by gel filtraton. A typical elution profile from an analytical column is shown in Figure 1. Analysis of the column fractions by SDS–PAGE shows that the first peak contains clathrin and clathrin light chains, which in 0.5 M Tris remain tightly associated (Figure 2). Polypeptides found in the second peak are known to promote the polymerization of clathrin triskelia (Keen *et al.*, 1979). The 180 kd polypeptide, which emerges just ahead of the bulk of the 100–110 kd assembly proteins was previously disregarded or assumed to be contaminating clathrin

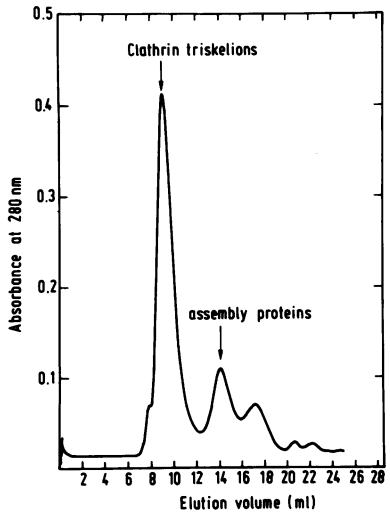

Fig. 1. Gel-filtration of solubilized coat protein. Purified coated vesicles were incubated with 0.5 M Tris-HCl, 2 mM EDTA, pH 7.0. Extracted peripheral membrane proteins were clarified by ultracentrifugation and then subjected to gel-filtration on a Superose 6 column connected to an f.p.l.c.-system.

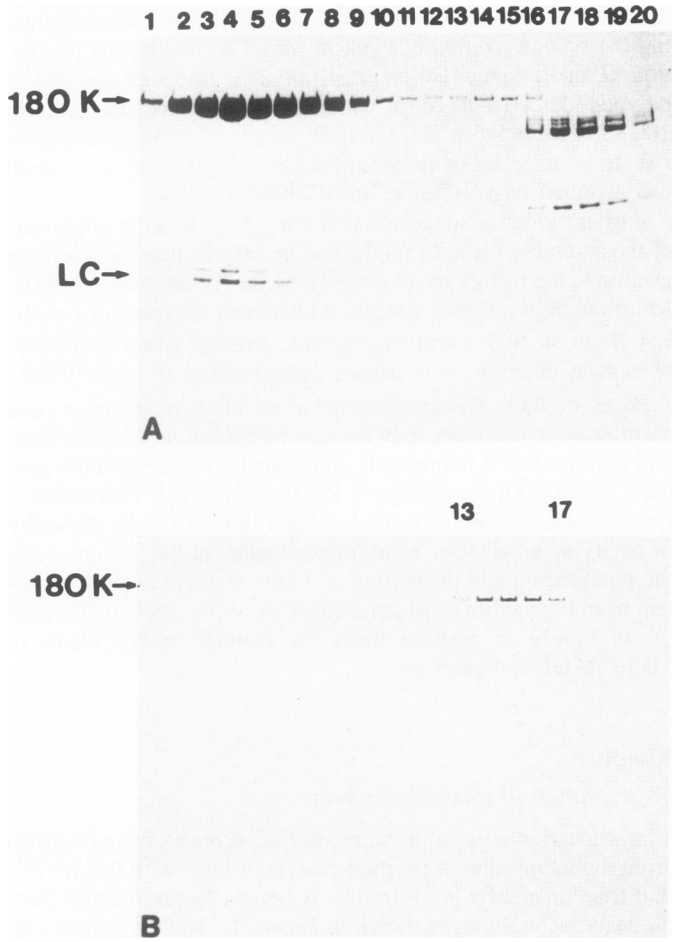

Fig. 2. Electrophoresis and immunoblot analysis of individual Superose 6 column fractions. Aliquots of fractions from the first and second peaks of the gel filtration elution were analysed by SDS−PAGE and the separated polypeptides were either stained for protein (**A**) or electro-blotted onto nitrocellulose paper and reacted with the monoclonal antibody $MAB_{AP180\text{-}I}$ (**B**). Note that the antibody does not stain the 180 kd clathrin heavy chain but a 180 kd polypeptide that elutes somewhat earlier than the bulk of the 100−110 kd assembly proteins.

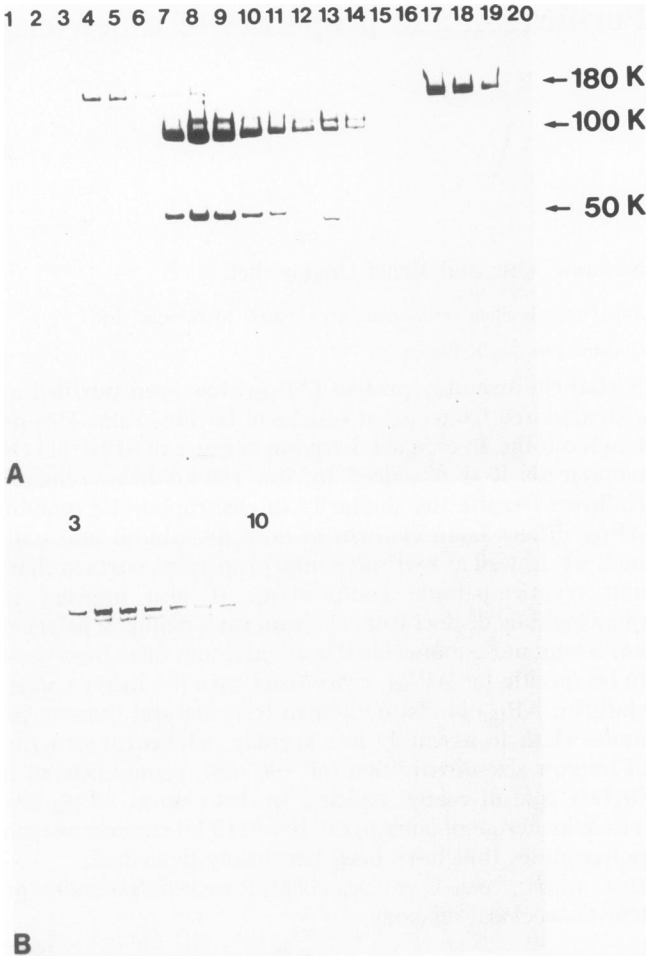

Fig. 3. Fractionation of assembly proteins by ion-exchange chromatography. Superose 6 column fractions that contained the new 180 kd protein and the 100−110 kd assembly proteins were pooled and after dialysis against 20 mM ethanolamine, 2 mM EDTA, pH 9.0, applied to a Mono Q anion exchange column. The column was eluted with a linear 0−0.5 M sodium chloride gradient (see Materials and methods for details). Protein-containing fractions were analysed by SDS−PAGE and the gels were either stained for protein (**A**) or analysed by immuno-blotting techniques with $MAB_{AP180\text{-}I}$ (**B**). The 180 kd polypeptide began to elute at 0.18 M sodium chloride, with extraneous clathrin triskelia added to the assembly proteins prior to the Mono Q-run, eluted at 0.35 M salt. $MAB_{AP180\text{-}I}$ also recognizes a 170 kd polypeptide that probably represents a proteolytic derivative of the 180 kd polypeptide.

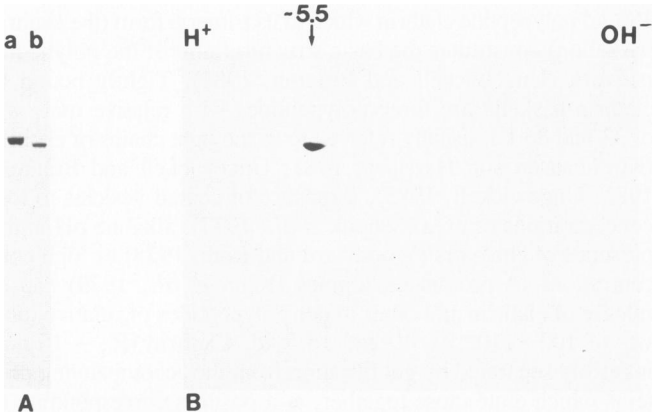

Fig. 4. Analysis of the 180 kd protein by one- and two-dimensional gel electrophoresis. A light loading of clathrin (**track a**) was run in a 10% polyacrylamide gel, together with the 180 kd protein (**track b**). Note that the 180 kd protein migrates slightly ahead of the clathrin heavy chain and it focuses as a single spot with an apparent pI of 5.5 (**B**).

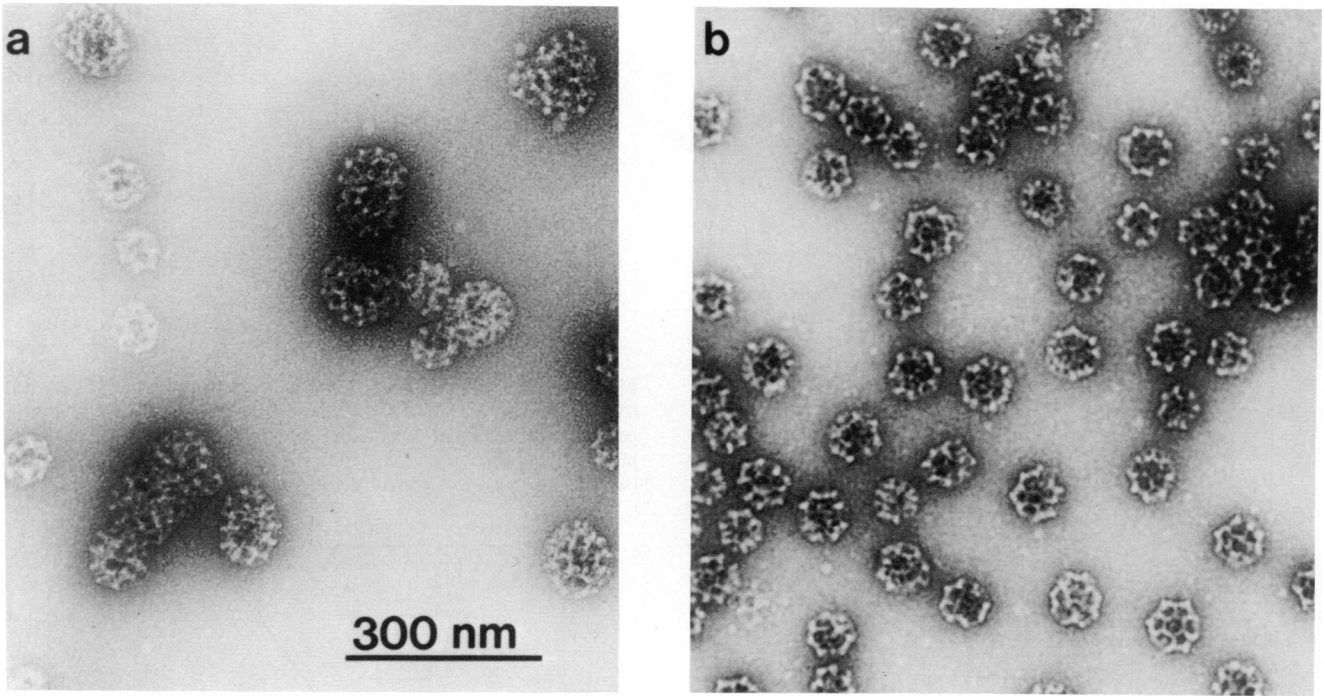

Fig. 5. Electron microscopy of reassembled cages. Clathrin triskelia were reassembled either in the presence of 2 mM calcium (**a**) or the 180 kd protein (**b**) (see Materials and methods for details). Note that the cages in (**b**) are smaller in size and more uniform in diameter.

(Keen, 1985). This interpretation became open to question when the column fractions were screened by immunoblotting with a monoclonal antibody ($MAB_{Ap180\text{-}I}$) which was initially supposed to be directed against the clathrin heavy chain. Contrary to expectation $MAB_{Ap180\text{-}I}$ reacted only with the 180 kd polypeptide, eluting just in front of the assembly proteins (Figure 2B).

Fractions containing the 180 kd protein and the 100–110 kd assembly proteins were pooled and subjected to ion-exchange chromatography on a Mono Q anion exchange column in conjunction with a fast protein liquid chromatography system. The 180 kd protein eluted from the column at 0.18 M NaCl, while residual clathrin together with its associated light chains emerged only at 0.35 M salt (Figure 3A). Light chain-free clathrin elutes somewhat earlier at about 0.3 M NaCl (not shown). The 100–110 kd assembly protein complexes appeared, partially resolved, in the region of the elution profile between 0.2 and 0.26 M NaCl. The 180 kd protein could be further purified by either adsorption chromatography on hydroxyapatite which removed residual 100–110 kd assembly proteins or, by gel filtration, which eliminated proteolytic products of the 180 kd protein (not shown). The 180 kd protein was observed to be highly susceptible to proteolysis, which initially reveals itself in the appearance of a band of 170 kd. This fragment is also recognized by $MAB_{Ap180\text{-}I}$ (Figure 3B). When purified 180 kd protein and clathrin, at low loading, were applied to adjacent lanes of a 5–15% polyacrylamide gradient SDS gel, the 180 kd protein was seen to migrate perceptibly faster (Figure 4A). Isoelectric focusing of the 180 kd protein in gels containing 9.5 M urea led to a single zone at a pI of ~5.5 (Figure 4B). So far, it has not been possible to focus the clathrin heavy chain in polyacrylamide gels.

Function of the 180 kd protein

To establish whether the new protein is indeed a constituent of

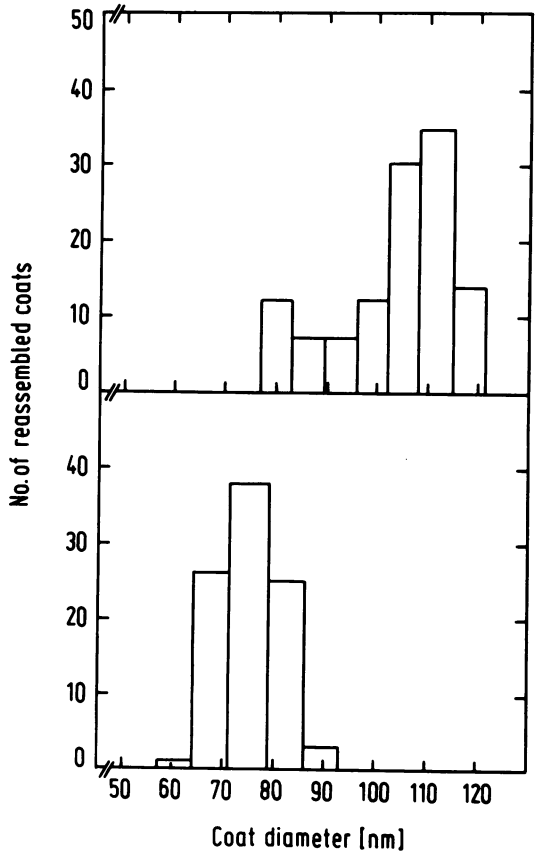

Fig. 6. Histogram of diameters of coats, reassembled either in the presence of calcium (**upper panel**) or of the purified 180 kd polypeptide (**lower panel**).

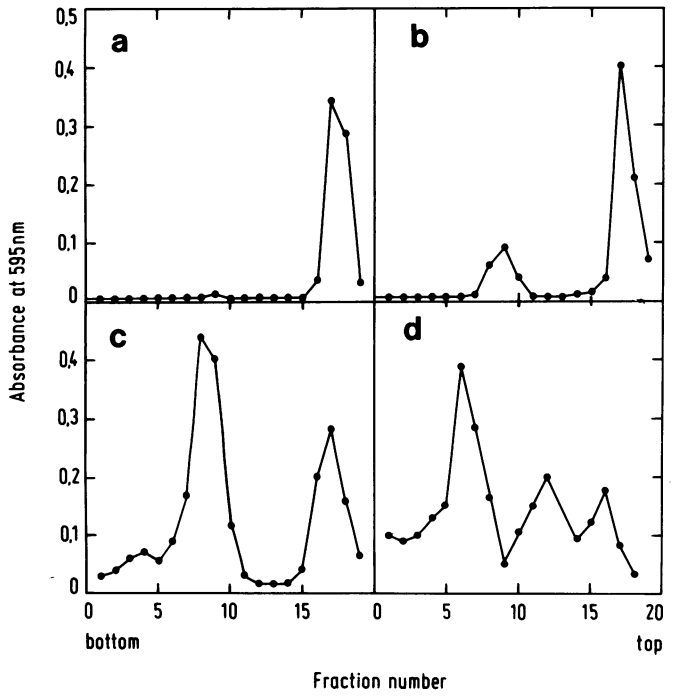

Fig. 7. Evaluation of clathrin reassembly by sucrose density centrifugation. (**a**) 60 μg purified clathrin alone after dialysis against calcium-free assembly buffer; (**b**) clathrin assembled in the presence of 4 μg 180 kd polypeptide; (**c**) clathrin assembled in the presence of 16 μg 180 kd protein; (**d**) clathrin alone after dialysis against calcium-containing assembly buffer.

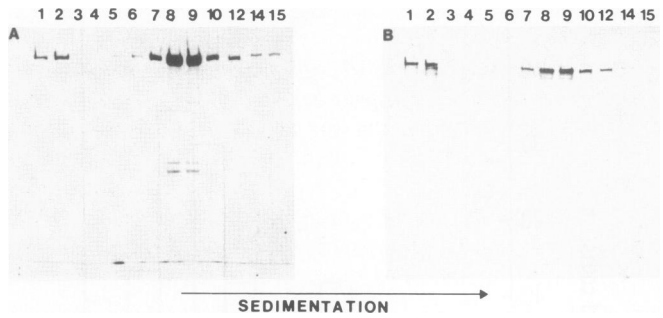

Fig. 8. Interaction of the 180 kd polypeptide with reassembled clathrin cages. Clathrin (60 μg) was reassembled in the presence of 15 μg 180 kd protein and then subjected to sucrose density centrifugation. Individual fractions were analysed by SDS–PAGE. (**A**) shows an electrophoretic gel stained for protein and (**B**) a nitrocellulose replica after exposure to $MAB_{AP180\text{-}I}$ to reveal only the 180 kd protein. The majority of the 180 kd protein co-sedimented in this experiment with clathrin cages.

coated vesicles and not merely an adventitious contaminant in our coated vesicle preparations, the 180 kd protein was tested for an effect on the assembly of purified clathrin. For this experiment, clathrin triskelia were mixed with increasing proportions of the 180 kd protein in 0.5 M Tris and then dialysed for at least 16 h against 20 mM MES-buffer at pH 6.5. As controls, clathrin triskelia alone were dialysed against the same buffer in the presence and absence of millimolar calcium ion concentrations. Assembly was monitored by electron microscopy and for purposes of quantitation by sucrose density centrifugation of the incubation mixture. The electron micrograph in Figure 5a shows a representative field of cages, that were reconstituted in the presence of calcium ions. The broad size distribution of these structures is apparent from the histogram in Figure 6 and also

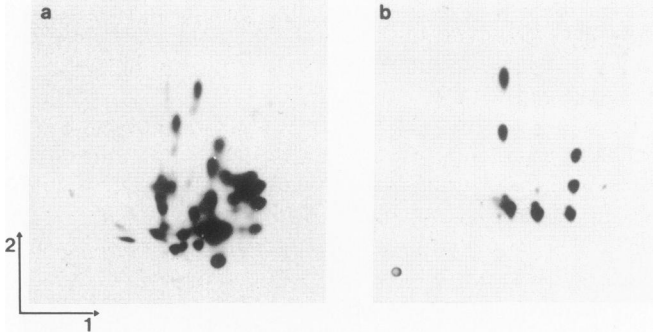

Fig. 9. Autoradiographs of two-dimensional peptide maps of ^{125}I-labelled tryptic peptides from the clathrin heavy chain (**a**) and AP_{180} (**b**). Tryptic peptides were separated on cellulose thin layer plates by electrophoresis at pH 3.5 in the first dimension and ascending chromatography in the second (see Materials and methods for details).

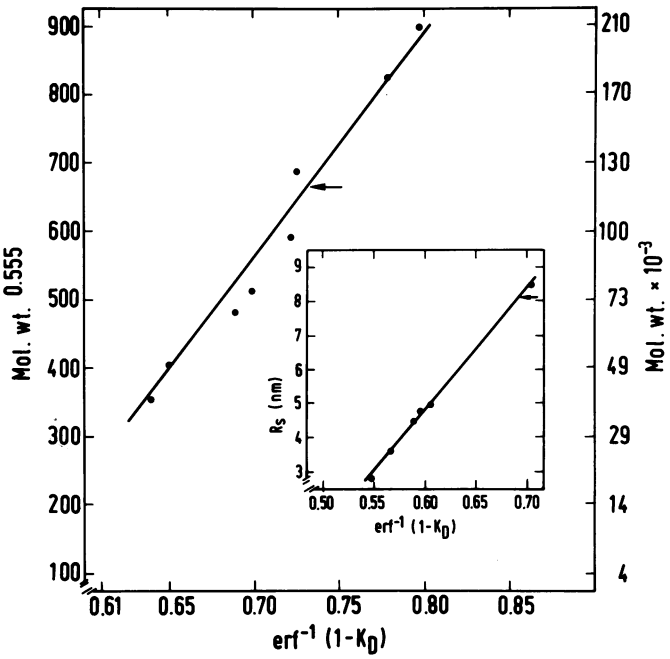

Fig. 10. Mol. wt and Stokes radius of AP_{180}. 40 μg unfolded AP_{180} in 6 M guanidinium-HCl, 2 mM EDTA, 2% β-mercaptoethanol were applied to a Superose 6 gel-filtration column equilibrated with the same medium. The column was calibrated with reduced and unfolded myosin heavy chain (210 000), clathrin heavy chain (180 000), β-galactosidase (130 000), phosphorylase *b* (100 000), transferrin (77 000), BSA (69 000), mouse IgG heavy chain (50 000) and aldolase (40 000). The arrow denotes the elution position of AP_{180} which corresponds to an apparent mol. wt of 123 000. The apparent Stokes radius (R_S) of native AP_{180} was determined by gel-filtration on Superose 6 in 0.5 M Tris-HCl, 2 mM EDTA, pH 7.0 (inset). The column was calibrated with thyroglobulin (8.5 nm), catalase (5 nm), aldolase (4.8 nm), liver alcohol dehydrogenase (4.5 nm), BSA (3.6 nm) and ovalbumin (2.9 nm). The elution volume of AP_{180} corresponds to an apparent Stokes radius of 8.1 nm. K_D: Partition coefficient for the protein between the mobile and stationary phases.

from their sedimentation profiles (Figure 7d). In contrast, cages assembled in the presence of the 180 kd protein but without calcium measure < 100 nm in diameter (Figure 5b) and their size distribution is rather uniform (Figure 6). No ordered self-assembly of clathrin triskelia occurs in the absence of calcium ions (Keen *et al.*, 1979) or of the 180 kd protein (Figure 7a). To demonstrate the co-sedimentation of the 180 kd protein with the reconstituted cages, fractions from a sucrose gradient were

analysed by SDS−PAGE followed by blotting onto nitrocellulose paper. By probing the paper with $MAB_{Ap180\text{-}I}$ it was demonstrated that a large proportion of the 180 kd protein sedimented with the reconstituted clathrin cages (Figure 8).

The above results strongly suggest that the 180 kd protein is a clathrin assembly protein which modulates the assembly of cages in a similar manner to the assembly polypeptides (at least four in number) of the HA-II group (Pearse and Robinson, 1984). The assembly-promoting activity of the 180 kd protein, which will henceforth be referred to as AP_{180} was completely destroyed after heating for 5 min at 90°C. AP_{180} was also successfully purified from crude coated vesicle extracts by affinity chromatography on $MAB_{Ap180\text{-}I}$ immobilized by covalent attachment to Sepharose beads. Bound AP_{180} was eluted with diethylamine, pH 11.4, and the recovered material was shown to promote the self-assembly of clathrin triskelia (not shown). It therefore appears that the promotion of assembly is an inherent property of AP_{180} and does not arise from an unresolved contaminant in the preparation.

Biochemical and biophysical characterization of AP_{180}

The similar electrophoretic mobility of AP_{180} to that of the clathrin heavy chain prompted comparison of the two-dimensional tryptic peptide maps of clathrin, AP_{180} and the presumed proteolytic derivative of AP_{180}, migrating at 170 kd (see Figure 3). The polypeptides were purified by SDS−PAGE either before or after radioiodination and were then digested to completion with trypsin. Figure 9 shows that there are no obvious similarities between the peptide maps of AP_{180} and clathrin whereas those of AP_{180} and the 170 kd polypeptide were identical (not shown). This proves that the latter is related to AP_{180} and both are unrelated to clathrin. The surprisingly simple fingerprint of AP_{180} could be explained by the results of the amino acid analysis of AP_{180}, which indicated that AP_{180} contains at most only 10 tyrosine residues. Tyrosine is known to be the prime target of the iodination reaction (Hunter and Greenwood, 1962).

Gel filtration in 0.5 M Tris gave an apparent Stokes radius of 8.1 nm for AP_{180} (Figure 10) and sucrose density gradient centrifugation in the presence of suitable markers yielded an approximate sedimentation coefficient of 3.5S (not shown). From these data and a partial specific volume of 0.73 ml/g, calculated from the amino acid composition, a mol. wt of 119 000 was obtained for AP_{180}. The gross discrepancy between this value and that inferred from SDS−PAGE led us to determine the polypeptide mol. wt of AP_{180} by gel filtration in 6 M guanidine hydrochloride (Mann and Fish, 1972), a more securely based method than either of the others. The elution volume of the reduced, unfolded polypeptide corresponded to a mol. wt of 123 000 (Figure 10) which is in fairly good agreement with the hydrodynamic value obtained for the native protein. Taken together, these results indicate first that the electrophoretic mobility in the SDS gel gives an incorrect mol. wt for AP_{180} and second that AP_{180} behaves as a monomer in 0.5 M Tris.

Discussion

The new assembly protein AP_{180}, described above, was discovered in the course of the characterization of the monoclonal antibody $MAB_{Ap180\text{-}I}$. The spleen cells used for the fusion originated from a mouse that had been immunized with total Tris-soluble coated vesicle protein from bovine brain. On nitrocellulose gel replicas the IgG $MAB_{AP180\text{-}I}$ reacts specifically with a polypeptide of M_r 180 000. This protein has been purified and shown to differ from clathrin in respect of its hydrodynamic radius, sedimentation coefficient, mol. wt, tryptic peptide map and isoelectric point. Furthermore, polyclonal rabbit antisera against AP_{180} do not cross-react with clathrin and *vice versa* (data not shown). On the basis of these results it can be ruled out that the AP_{180} represents a modified clathrin heavy chain. The reason for the abnormal electrophoretic mobility of AP_{180} in SDS is not yet understood. However, anomalous SDS binding can result from glycosylation, from a high degree of phosphorylation or from clusters of amino acids of like charge (Williams and Gratzer, 1971; Bretscher, 1971). In the present case phosphorylation is an unlikely explanation because AP_{180}, unlike known phosphoproteins (Bernardi and Kawasaki, 1968), adsorbs only weakly to hydroxyapatite (not shown).

AP_{180} is likely to be a true coated vesicle constituent, because it not only co-purifies with coated vesicles but also interacts *in vitro* with clathrin triskelia and promotes their assembly into 70−80 nm cage-like structures. AP_{180} differs in its properties from the so-called HA-II group of the 100−110 kd assembly proteins, isolated by adsorption chromatography on hydroxyapatite (Pearse and Robinson, 1984). The HA-II group consists of at least three polypeptides, with mol. wts between 100 and 110 kd, two of which are barely resolved by SDS−PAGE, and a 50 kd ATP-binding protein which becomes auto-phosphorylated *in vitro* (Pauloin and Jolles, 1984; Campbell *et al.*, 1984) but not apparently *in vivo* (Keen and Black, 1986). According to the results of chemical crosslinking studies, the 50 kd polypeptide and the 100−110 kd polypeptides associate to complexes of some 300 kd (Pearse and Robinson, 1984). In contrast, AP_{180}, at least in 0.5 M Tris, behaves as a monomer, and its Stokes radius of 8.1 nm which exceeds that of the HA-II group assembly polypeptides by about 1 nm suggests an elongated or perhaps highly flexible structure for AP_{180}.

Electron microscopic views of cages that had been reconstituted in the presence of AP_{180} appear less densely packed with protein than those formed under the action of the HA-II group assembly proteins (compare Figure 5b with Figure 7 of Pearse and Robinson, 1984). This difference in appearance could be explained by the three times greater molar mass of the HA-II polypeptide complex, compared to AP_{180} (300 kd against 120 kd) and the tendency of HA-II polypeptides themselves to form large aggregates under reconstitution conditions (not shown).

Since AP_{180} and the polypeptides of the HA-II group affect the polymerization of clathrin triskelia in a similar manner, it might reasonably be supposed that they also share identical or at least overlapping binding sites on the clathrin heavy chain. Earlier work indicated that the polypeptides of the HA-II group do interact with the triskelion core domain, which lacks the distal 50 kd globular leg segments, that are known to be unnecessary for self-assembly of clathrin *in vitro* (Ungewickell, 1984). In principle, the binding site for AP_{180} on clathrin triskelia could be identified by determining whether AP_{180} competes on clathrin with the HA-II group or by examining its binding to fragments of clathrin. However, it is already known that the light chains are not required for the binding of AP_{180} to clathrin (not shown).

At present definitive data on the stoichiometry of the interaction beween clathrin and AP_{180} is still lacking. These results indicate, however, that one molecule of AP_{180} per clathrin triskelion suffices to induce nearly complete polymerization of the latter (Figure 8). Thus the assembly protein may need only to generate a nucleus of associated triskelia for uniform assembly to proceed rapidly to completion. It is also conceivable that the effect of AP_{180} is not a kinetic one, and that a single molecule

of AP_{180} for each edge of the facets of the cages may be sufficient to shift the equilibrium towards the (cooperatively) associated state. A hexagonal facet, for example, is made up of six triskelia, and would therefore be expected to accommodate the same number of assembly protein molecules.

Compared to the 100–110 kd assembly proteins AP_{180} appears to be only a minor component (~10%) of the assembly protein fraction. However, it should be considered that the former resolve into six polypeptides by combination of hydroxyapatite chromatography (in the presence and absence of SDS) and SDS–PAGE. Based on immunological studies and the results of one-dimensional peptide mapping, Robinson and Pearse (1986) arrived at the conclusion that there are distinct differences between the different 100–110 kd polypeptides and that they are therefore unlikely to represent proteolytic derivatives of a common precursor. When two-dimensional tryptic- and chymotryptic fingerprints of AP_{180} and the other assembly polypeptides were compared no obvious similarities could be discerned between any of them (data to be published). There is also recent evidence for a specific association of particular 100–110 kd assembly proteins with coated membranes from different intracellular locations. Two polyclonal antisera, that were shown by Robinson and Pearse (1986) to recognize different subsets of the 100–110 kd polypeptides on nitrocellulose gel replicas, also gave rise to distinguishable immunofluorescent staining patterns in tissue culture cells. One serum reacted preferentially with antigens in the Golgi region of the cells, while the other gave stronger staining of the plasma membrane than the Golgi. This suggests that the different assembly polypeptides may reside in different organelles. Such differential intracellular distribution of assembly proteins may also reflect the different compositions of the coated membranes in respect of their cargo molecules. Two lines of evidence suggest that the assembly proteins may interact directly with membrane proteins. First, controlled proteolysis of the 100–110 kd polypeptides abolishes the ability of clathrin triskelia to bind to the membrane of coated vesicles (Unanue *et al.*, 1981) and second, polypeptides of the HA-II group were shown to interact with the purified mannose-6-phosphate receptor (Pearse, 1985). The fact that the transferrin receptor failed to interact under similar conditions with the HA-II group of polypeptides could reflect their specificity for particular cargo molecules. The cytoplasmic domains of receptors are most likely to interact with components of coated pits. In the case of the LDL-receptor this conjecture has indeed proved correct (Lehrman *et al.*, 1985). When the primary structures of the cytoplasmic domains of five receptors, which are known to become sequestered in coated pits, were compared, no obvious common structural motifs emerged (for review see Goldstein *et al.*, 1985). Therefore an adaptor that will link a receptor to the principal coat protein clathrin may be postulated. The assembly proteins might perhaps serve such a function. In this case they would be expected to possess an invariant and a variable domain, the first containing the clathrin binding site, the second a site for the attachment of defined membrane proteins, destined for intracellular translocation. AP_{180} which can be obtained pure with relative ease, is probably very well suited for the exploration of such possibilities, initially by way of standard procedures, such as controlled proteolytic dissection of functional domains.

Materials and methods

Materials

Bovine brains from freshly slaughtered animals were obtained from a local slaughterhouse and processed within 1 h of slaughter. Gel filtration media (Superose 6 and Sepharose CL 4B), Mono Q ion exchange resin, protein standards and CNBr-activated Sepharose 4B were obtained from Deutsche Pharmacia, Freiburg (FRG); t.l.c. cellulose plastic sheets without fluorescent indicator were obtained from Merck, Darmstadt (FRG); ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N'N'*,-tetraacetic acid (EGTA), 2-(*N*-morpholino)ethanesulfonic acid (MES), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF) from Sigma, St. Louis (USA); carrier-free I^{125} (3.7GBq/ml) from Amersham Buchler GmbH, Braunschweig (FRG); Ampholytes and Coomassie Brilliant Blue from LKB Instrument GmbH, Gräfelfing (FRG); Fuji RX X-ray film from Fuji Film Co., Ltd (Japan); Bradford reagent from Bio-Rad Laboratories GmbH, Munich (FRG); peroxidase-conjugated rabbit IgGs to mouse immunoglobulins from Dakopatts GmbH, Hamburg (FRG).

Methods

Purification of clathrin and assembly proteins. Coated vesicles from bovine brain tissues were prepared essentially according to the procedure of Keen *et al.* (1979) and stored frozen in aliquots at −70°C in a buffer containing 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM DTT, pH 6.5. Clathrin and other peripheral membrane proteins were liberated from the coated vesicle membrane during a 15-min incubation at 37°C in a medium containing 0.5 M Tris-HCl, 50 mM MES, 0.25 mM $MgCl_2$, 0.5 mM EGTA, 1 mM ethylene diaminetetraacetic acid (EDTA), 1 mM DTT, 0.1 mM PMSF, pH 7.0. The extract was clarified by ultracentrifugation at 100 000 *g* for 45 min. Prior to gel filtration on an analytical Superose 6 f.p.l.c. column the extract was concentrated by centrifugation in a Centricon-30 microconcentrator to a protein concentration of a 4–6 mg/ml. Up to 0.4 ml of the extract was applied to a 10 × 300 mm Superose 6 column, used in conjunction with a f.p.l.c. system. The column was eluted with 0.5 M Tris-HCl, 2 mM EDTA, 1 mM DTT, 0.02% NaN_3 and run at a flow rate of 0.5 ml/min. Fractions containing clathrin triskelia and the assembly proteins were separately pooled and dialysed overnight against 20 mM ethanolamine, pH 9.0, 2 mM EDTA and 1 mM DTT. Up to 5 mg clathrin was applied to a Mono Q HR 16/10 anion-exchange column and eluted with a linear 0–0.5 M NaCl gradient. The total gradient volume was 30 ml and the flow rate 0.5 ml/min. Clathrin eluted together with its light chains but free of assembly proteins at ~0.35 M NaCl. Clathrin-containing fractions were pooled and the clathrin triskelia were either used immediately for reconstitution experiments with AP_{180} (see below) or assembled into cages by dialysis against 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM DTT, 2 mM $CaCl_2$, pH 6.5. The cages were rapidly frozen in liquid nitrogen and stored in aliquots at −70°C. The assembly proteins were fractionated on the same column under identical elution conditions. Aliquots of all fractions were analysed by SDS–PAGE. Fractions that contained the partially separated assembly proteins were pooled separately. For some experiments AP_{180} was further purified by hydroxyapatite adsorption chromatography on a 5 × 50 mm column connected to the f.p.l.c.-system. The hydroxyapatite was prepared according to Spencer (1978) and equilibrated in 0.5 M Tris-HCl, 2 mM K/PO_4, pH 7.0. AP_{180}-containing fractions were applied directly to the hydroxyapatite column and AP_{180} was eluted with a linear 2–500 mM K/PO_4 gradient. AP_{180} eluted in electrophoretically pure form in a narrow peak at ~20 mM phosphate. The 100–110 kd assembly proteins of the HA-II group eluted at 0.2 M phosphate. Purified AP_{180} was concentrated when necessary by centrifugation in a Centricon-30 microconcentrator and stored in the presence of NaN_3 at 4°C.

Stokes radius and molecular weight determinations. The Stokes radii of native assembly proteins were determined in 0.5 M Tris–HCl, 2 mM EDTA, 1 mM DTT, pH 7.0, by gel filtration on a 10 × 300 mm Superose 6 column, calibrated with appropriate marker proteins of known Stokes radii. The void volume of the column was determined with intact cages that had been fixed with glutaraldehyde, and the included volume was determined with ATP. Elution volumes were determined by monitoring the eluant at 280 nm. For molecular weight determinations of completely unfolded assembly proteins the same column was equilibrated in 6 M guanidine-HCl, 2 mM EDTA, 50 mM Tris-HCl, 2 mM DTT, pH 7.5. Lyophilized marker proteins were dissolved in column buffer containing instead of DTT 2% β-mercaptoethanol as reducing agent and briefly warmed to 37°C prior to gel filtration. AP_{180} was first dialysed against 0.1 M ammonium bicarbonate and then lyophilized and redissolved in column buffer containing 2% β-mercaptoethanol. Void- and included volumes were determined as described above. AP_{180}-containing fractions were checked for possible degradation of the protein by SDS–PAGE after most of the guanidinium salt had been removed by dialysis against 8 M urea, 2 mM EDTA, 50 mM Tris-HCl, pH 7.5. Before electrophoresis the protein was concentrated by precipitation with trichloroacetic acid (5.5% final concentration).

Clathrin assembly assay. For assembly protein-dependent cage formation AP_{180} (2–22 μg) were added to 60 μg of clathrin triskelia in a final volume of 0.4 ml and dialysed overnight at 4°C against 20 mM MES, 2 mM DTT, pH 6.5 (assembly buffer). To test clathrin assembly in the absence of assembly proteins 60 μg clathrin was dialysed against either assembly buffer or assembly buffer supplemented with 2 mM $CaCl_2$. Aliquots of the incubation cocktail were negatively stained with uranyl acetate according to the procedure of Valentine *et al.* (1968)

and examined in a Philips EM 300 at an acceleration voltage of 80 kV. To estimate the extent of assembly, 0.3 ml aliquots were analysed by centrifugation in 4.5 ml 5–30% sucrose gradients made up in assembly buffer with or without $CaCl_2$. The gradients were centrifuged for 1 h at 4°C in a Beckman SW 60 rotor at 37 000 r.p.m. 0.2 ml fractions were collected manually after puncturing the bottom of the centrifuge tubes. The protein concentration of the fractions was determined according to the procedure of Bradford (1976).

Antibody production. Female BALB/c mice 6–8 weeks old were immunized at intervals of 3 weeks with the membrane protein fraction that had been extracted with 0.5 M Tris–HCl, 2 mM EDTA, pH 7.0 from purified bovine brain coated vesicles; Freund's complete adjuvant was used with the first injection and incomplete adjuvant for three subsequent injections. Sera were tested by immunoblotting using total coated vesicle protein. Spleen cells from the mouse with the strongest reaction to the major coated vesicle proteins were fused with cells from the myeloma line PAI (Stocker, 1982) according to the procedure of Debus *et al.* (1983). After fusion the cells were distributed between fifteen 24-well plates and cultured in HAT-medium. The medium was changed twice a week. Supernatants were tested by the peroxidase spot test (Hawkes *et al.*, 1982). Positive supernatants were subsequently tested by immunoblotting. Colonies of interest were sub-cloned by limited dilution. Ascites fluids were produced in female BALB/c mice and IgGs were purified from ascites fluid by ion-exchange chromatography on Mono Q (Clezardin *et al.*, 1985).

Purification of AP_{180} by immuno-affinity chromatography. A crude vesicle preparation from 200 g bovine brain tissue (referred to as fraction C by Keen *et al.*, 1979) was extracted with 0.5 M Tris-HCl, 2 mM EDTA, 1 mM DTT, pH 7.0, and the extract was clarified by ultracentrifugation at 100 000 *g*. The supernatant was dialysed against 0.05 M triethanolamine, 100 mM NaCl, 2 mM EDTA, pH 8.1 and then cycled twice through a column containing 20 mg of purified $MAB_{AP180\text{-}I}$, covalently attached to 10 ml Sepharose 4B. The column was washed with 20 ml of PBS, 0.4 M NaCl in PBS and PBS, respectively. Approximately 1 mg AP_{180} was eluted with 0.05 M diethylamine, pH 11.4. 2.5-ml fractions were collected in tubes that already contained 0.3 ml 1 M Tris-HCl, pH 7.0, in order to minimize the possibility of irreversible damage to the protein by the highly alkaline pH.

Determination of sedimentation coefficient. The sedimentation coefficient of AP_{180} was estimated by sucrose density gradient centrifugation in the presence of suitable marker proteins of known sedimentation coefficients (Siegel and Monty, 1966). Thirty micrograms of the protein in 0.5 M Tris-HCl, 2 mM EDTA, pH 7.0, was loaded together with catalase (11.3S), fumarase (9.2S), BSA (4.6S) and myoglobin (2S) on a 4.5-ml 5–20% linear sucrose gradient made up in the Tris-buffer. The gradients were centrifuged for 20 h at 50 000 c.p.m. in a Beckman SW 60 rotor. The tubes were punctured and 0.2-ml fractions were collected manually. The protein composition of each fraction was analysed by SDS–PAGE and by immunoblotting.

Peptide mapping. Two-dimensional maps of ^{125}I-labelled tryptic peptides were obtained by a modification of the procedure of Elder *et al.* (1977). Purified clathrin and AP_{180} were iodinated with [^{125}I]NaI using chloramine T as the oxidant (Hunter and Greenwood, 1962). Fifteen micrograms of clathrin and AP_{180}, respectively, were first denatured by boiling in 30 μl SDS-sample buffer (Laemmli, 1970) from which reducing reagents and bromophenol blue had been omitted. Then 11.1 MBq[^{125}I]NaI and 4 μl chloramine T (1 mg/ml in 0.05 M sodium phosphate buffer pH 7.5) were added. The reaction was terminated after 3 min by addition of 3 μl of a 1 M DTT stock solution, 5 μl β-mercaptoethanol and 3 μl sodium iodide (1 mg/ml). After addition of 15 μl of buffer of twice the concentration of the SDS-sample buffer the samples were briefly boiled and then subjected to electrophoresis in 10% polyacrylamide gels. The polypeptides were lightly stained with Coomassie Brilliant Blue, excised from the gel, destained completely, equilibrated in 50 mM NH_4HCO_3 and then lyophilized. The gel pieces were incubated for 24 h at 37°C with 1 ml 50 mM NH_4HCO_3, containing 0.05 mg/ml trypsin. The digest was lyophilized and redissolved in 10–20 μl acetic acid/formic acid/water, 15/5/80, v/v/v, pH 3.5. The peptides were spotted onto cellulose thin layer plates and separated by electrophoresis at pH 3.5 in the first dimension and subjected to ascending chromatography using butanol/pyridine/acetic acid/water, 65/50/10/40, v/v/v/v in the second dimension. Autoradiography was performed at room temperature with pre-flashed X-ray film.

Miscellaneous techniques. SDS-PAGE was performed according to Laemmli (1970) and two-dimensional gels according to the procedure of O'Farrell (1975) using ampholytes with a nominal pH range of 3.5–10 (actual pH range: 4.2–8.3) in the first dimension. For the second dimension the focussed proteins were electrophoresed in a 10% SDS–polyacrylamide gel and then stained for protein with Coomassie Brilliant Blue.

Immunoblotting experiments were performed as described in detail previously (Ungewickell, 1985). The concentration of AP_{180} was determined spectrophotometrically and calculated from the relation: c (mg/ml): 0.144 ($A_{125nm} - A_{225nm}$) (Wadell, 1956). The amino acid composition of AP_{180} was determined after 22 h hydrolysis in 6 M HCl at 110°C in a Biotronic amino acid analyser.

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